STIC-ILL

Vol 10

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Sent: Tuesday, April 17, 2001 7:47 PM
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342083

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1. Chong, M. W. et al., Int. J. Pharmaceutics (Jun 1998) 167(1-2): 25-36
2. Chen, B.-L. et al. J. Pharmaceutical Sciences (1994) 83(12): 1657-1661
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Aggregation Pathway of Recombinant Human Keratinocyte Growth Factor and Its Stabilization

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Received February 2, 1994; accepted June 6, 1994

Recombinant human keratinocyte growth factor (rhKGF) is prone to aggregation at elevated temperatures. Its aggregation pathway is proposed to proceed initially with a conformational change which perhaps results from repulsion between positively charged residues in clusters forming heparin binding sites. Unfolding of the protein leads to formation of large soluble aggregates. These soluble aggregates then form disulfide cross-linked precipitates. Finally these precipitates are converted to scrambled disulfides and/or non-disulfide cross-linked precipitates. Stabilizers such as heparin, sulfated polysaccharides, anionic polymers and citrate can greatly decrease the rate of aggregation of rhKGF at elevated temperatures. These molecules may all act by reducing charge repulsion on the protein thus stabilizing the native conformation. EDTA, on the other hand, is found to inhibit disulfide formation in aggregates and has only a moderate stabilizing effect on rhKGF.

KEY WORDS: Keratinocyte Growth Factor; Aggregation Pathway; Protein Formulation; Protein Stability.

INTRODUCTION

Keratinocyte growth factor (KGF) is the seventh cloned member of the heparin-binding (fibroblast) growth factor family (1). Unlike other FGF family members with potent mitogenic activity for a variety of target cells, its potency is specific in stimulating epithelial cell growth (2-3). Recombinant human KGF (rhKGF) has been cloned and expressed in *E. coli* and contains 163 amino acid residues. It has increased mitogenic activity over that of the natural growth factor (4).

The stabilities of acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) have been studied (5-7). Both are prone to acid and thermal inactivation. Addition of heparin and heparin-like molecules stabilizes these proteins against aggregation (5,8). The stabilizing mechanism has been proposed to be physical in nature (9-10). However, enhanced stability of bFGF has been also observed in cysteine-substituted analogs (11) and in cysteine chemically modified analogs (12). Thus chemical instability may also play an important role in the mechanism of degradation of these proteins.

Similar to aFGF and bFGF, rhKGF aggregates rapidly at elevated temperatures and is inactivated under acidic conditions. Heparin and a number of sulfated polysaccharides have also been found to stabilize rhKGF. The hKGF protein is a single chain polypeptide with five cysteines, four of which form two disulfide bridges and the remaining free cysteine is buried inside the protein (E. Hsu, B.-L. Chen, and W. C. Kenney, unpublished results). Thus rhKGF is a good model protein to study mechanism of aggregation induced by physical changes. Here we report our investigation of the mechanism of aggregation of rhKGF and its stabilization.

MATERIALS AND METHODS

The human keratinocyte growth factor (hKGF) gene was cloned by the polymerase chain reaction from human fibroblast RNA into an expression plasmid and transformed into host *E. coli* cells by standard protocols (13). Fermentation was performed under standard conditions and rhKGF was purified by ion-exchange and hydrophobic interaction chromatography columns.

All chemicals were either analytical or USP grade. Heparin and sulfated polysaccharides were purchased from Sigma Chemical Co. Poly(acrylic acid) and poly(methacrylic acid) were purchased from Polysciences, Inc. Gel filtration standard was purchased from Bio-Rad Laboratories, Inc.

Formulation sample preparation: rhKGF was prepared in different formulations by dialysis using Spec/Por 7 dialysis tubing (MW cutoff 3,500). Polysaccharides and polymers, if present, were added after dialysis into appropriate buffers. After adjusting the rhKGF concentration to 0.5 mg/ml, samples were filtered through Costar 0.2 micron filter units and 0.5 or 1 ml volumes were aliquoted into 3 c.c. type 1 glass vials in a sterile laminar flow hood. The vials were sealed with rubber stoppers (1888 Teflon, West Co.) and 13 mm flip-off aluminum seals were crimped in place. Finally, the vials were placed in incubators set at different temperatures.

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis): Non-reducing and reducing SDS polyacrylamide gel electrophoresis was carried out on the formulation samples following Laemmli's protocol (14). The precast 15% Daiichi SDS gels were purchased from Integrated Separation Systems.

UV/Vis absorbance: 150-200 ul of protein solution was loaded into a quartz glass suprasil 10-mm pathlength ultramicro-cell. UV/Vis absorbance measurement was carried out on a Hewlett Packard (HP) 8452A diode-array spectrophotometer. Protein concentrations were determined using $A^{0.1\%} = 1.16$ at 280 nm based on calculation from the amino acid composition (15).

High performance liquid chromatography: HPLC analysis of rhKGF samples were performed using a Hewlett Packard 1090 liquid chromatography system equipped with a HP 3D Chemstation for data acquisition. Protein species were detected by absorbance at 230 nm using a HP diode-array detector.

For ion-exchange HPLC (IEX-HPLC), samples were equilibrated after injection for 5 min in 70% buffer A (20 mM

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sodium phosphate at pH 8.0) and 30% buffer B (20 mM sodium phosphate at pH 8.0 and 1 M NaCl) in a Pharmacia Mono S HR 5/5 glass column. Then a gradient was applied increasing from 30% buffer B to 80% buffer B in 30 min at a flow rate of 1 mL/min. Under these elution conditions, at least five rhKGF species are separable. These include a charge-changed degradation species, a monomer, a dimer, a trimer and a tetramer in order of elution off the column.

For size-exclusion HPLC (SEC-HPLC), samples were loaded into a Phenomenex BIOSEP-SEC-S2000 column (300 × 7.8 mm). Isocratic elution was applied at a flow rate of 1 mL/min using a buffer containing 100 mM sodium phosphate, pH 6.9, and 0.5 M sodium chloride. The rhKGF monomer, dimer, trimer and soluble aggregates are eluted as separate species.

Mitogenic bioassay: In vitro biological activity was determined by measurement of [^{3}H]-thymidine uptake by Balb/MK cells (2). Samples were first diluted in a bioassay medium consisting of 50% customer-made Eagle's MEM, 50% customer-made F12, 5 ug/ml transferrin, 5 ng/ml sodium selenite, 0.005% HSA and 0.005% Tween 20. Samples were then added into Falcon primeria 96-well plates seeded with indicator cells. Incorporation of [^{3}H]-thymidine during DNA synthesis was measured and converted to input rhKGF concentration by comparison to a rhKGF standard curve.

Acid lability: Solutions of rhKGF in the presence or absence of 1:10 (w/w) dextran sulfate (MW 500,000) were adjusted to pH 1.6 to 7. Samples were incubated at room temperature for 30 min and were then diluted 5,000 to 40,000 fold directly into the bioassay medium. *In vitro* bioactivity of remaining rhKGF was measured by the mitogenic bioassay.

RESULTS

rhKGF aggregates rapidly at elevated temperatures. At 45°C, rhKGF protein in a neutral phosphate buffered saline solution aggregates rapidly with a half-life of several hours. The aggregates become visible precipitates and exhibit no *in vitro* bioactivity.

The Aggregation (Precipitation) Pathway

Thermal unfolding analysis of rhKGF protein by circular dichroism (CD) and ultra sensitive differential scanning calorimetry (DSC) shows that aggregation (precipitation) follows immediately after unfolding and leads to increased light scattering and irreversibility (B.-L. Chen and L. Narhi, unpublished results). For example, in CD experiments, the initial unfolding showing the change in the ellipticity is protein concentration independent, and the subsequent aggregation showing increased light scattering is strongly protein concentration dependent. The rate of aggregation (precipitation) is so rapid near the melting temperature (about 60°C) that the experiments to determine a mechanism were carried out at a relatively low temperature, 37°C, where the aggregation rate is much slower and can be more accurately followed.

Three formulation samples containing 0.5 mg/ml of rhKGF were prepared: P7N (10 mM sodium phosphate at pH 7 and 140 mM NaCl), C7N (10 mM sodium citrate at pH 7 and 140 mM NaCl), and P7NE [10 mM sodium phosphate at pH 7, 140 mM NaCl and 5 mM ethylenediaminetetraacetic acid (EDTA)]. One ml of each of the three formulations were filled into 3 cc glass vials and stored at 37°C. At t = 0, 39 hr, 88 hr, 13 days and 1 month, the vials were removed and examined for aggregation. 250 ul of protein solution was withdrawn from each vial and filtered through a Costar 0.22 micron Spin-X centrifuge filter unit. Filtration of the aged samples by the spin-X centrifuge units separates precipitates from soluble rhKGF and soluble aggregates and yields a visually clear protein solution. The filtrate was subsequently analyzed by both HPLC and SDS-PAGE. In order to compare with the filtered protein sample, unfiltered protein sample in each vial was also analyzed by SDS-PAGE. Results of SDS-PAGE for both filtered and unfiltered protein samples are shown in Figure 1 and results of IEX-HPLC of filtered protein samples are shown in Figure 2.

On non-reducing SDS gels (Fig. 1A, B and C), two protein bands are observed corresponding to rhKGF monomer and rhKGF disulfide-bonded dimer, respectively. On reducing SDS gels (Fig. 1D, E and F), only the monomer is observed. If the samples are treated with thiol blocking agents such as iodoacetate or 5,5′-Dithio-bis-(2-nitrobenzoic Acid) (DTNB) prior to mixing with non-reducing SDS sample buffer, only the monomer is observed. Thus the observed dimer band is an artifact of SDS-PAGE analysis. EDTA appears to inhibit formation of the dimer species during SDS-PAGE analysis, since much less dimer species is observed in the sample P7NE (Fig. 1C). In all non-reducing SDS gels (Fig. 1A, B, C), filtered and unfiltered samples show almost no difference in total protein band intensities, suggesting that precipitates cannot be dissolved by SDS molecules.

The total amount of rhKGF protein on non-reducing SDS gels decreases with incubation time, indicating that highly cross-linked precipitates that are not solubilized by SDS gradually accumulate. On reducing SDS gels these precipitates can be partially recovered at earlier time points with band smearing observed (unfiltered samples in Fig. 1D, E and F). But at later time points, the majority of the rhKGF protein that precipitates is not recoverable on the reducing SDS gels. This suggests the presence of a population of different disulfide-linked species in the precipitates. Precipitates formed initially may involve simple disulfide linkages and can therefore be readily dissolved by reducing SDS sample buffers. On the other hand, precipitates developed at later stages may involve more complicated disulfide linkages and/or non-disulfide cross-links that can not be readily dissolved by reducing SDS sample buffers. Through this analysis, stages of formation of different types of cross-linked precipitates can be separated.

Soluble rhKGF and soluble aggregates in the filtrates were quantified by integrating the corresponding peak areas on HPLC chromatograms. Very small amounts of charge-changed degradation species and rhKGF dimer, trimer and tetramer species were observed in these three samples. Because rapid aggregation follows unfolding immediately, as suggested by the CD experiments, we do not expect a large population of unfolded rhKGF. Therefore soluble rhKGF species in both IEX-HPLC and SEC-HPLC are probably mainly composed of the native protein. Soluble aggregates appear to have a mass at least several hundred thousand

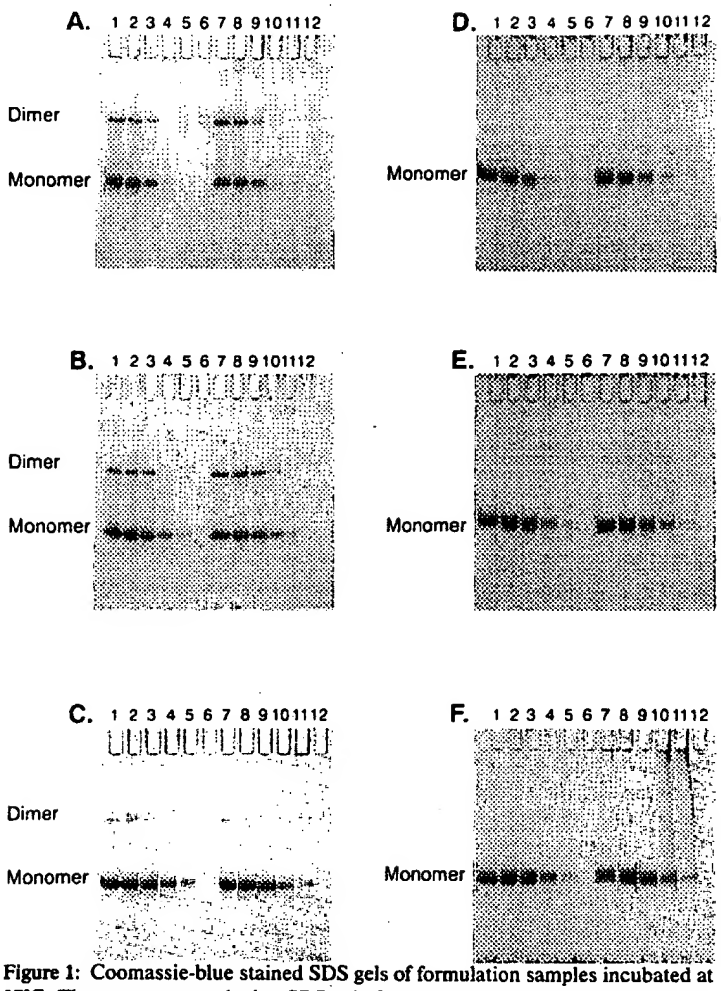

Figure 1: Coomassie-blue stained SDS gels of formulation samples incubated at 37°C. These are: non-reducing SDS gels for P7N (A), C7N (B) and P7NE (C); and reducing SDS gels for P7N (D), C7N (E) and P7NE (F). Lanes 1-5 are for filtered samples at t = 0, 39 hr, 88 hr, 13 days and 1 mo, respectively. Lanes 7-11 are for unfiltered samples at t = 0, 39 hr, 88 hr, 13 days and 1 mo, respectively.

daltons, calibrated by the Bio-Rad gel filtration standard, and elute at an earlier retention time than the monomer rhKGF species on SEC-HPLC. It was found that at 37°C a decrease in the amount of soluble rhKGF protein (due to aggregation which forms soluble aggregates and then precipitates) occurs rapidly with an apparent first order rate constant 0.376 day^{-1} for P7N and 0.160 day^{-1} for both C7N and P7NE (Fig. 2), respectively.

Soluble aggregates can be detected by SEC-HPLC. The kinetics of the appearance and the disappearance of these soluble aggregates for C7N and P7NE are shown in Figure 3. C7N and P7NE have the same apparent rate constants for the decrease of soluble rhKGF (Fig. 2). Soluble aggregates form at a similar rate in both formulations (see the increase in soluble aggregates in Fig. 3). However, the subsequent loss of soluble aggregates occurs at different rates in these two samples. Soluble aggregates of rhKGF exist longer in P7NE than in C7N, indicating that EDTA inhibits formation f disulfides or other cross-links in the soluble aggregates, thus inhibiting conversion of the soluble aggregates to the cross-linked precipitates.

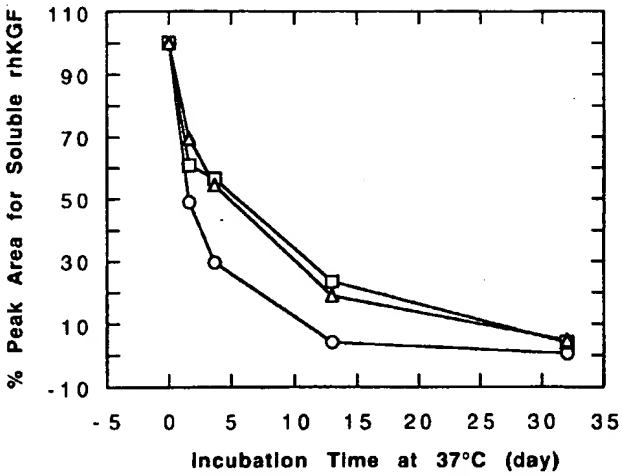

Figure 2: Kinetic curves of the percentage remaining soluble rhKGF of formulation samples P7N (○), C7N (△) and P7NE (□). Remaining soluble rhKGF was quantified by IEX-HPLC and is plotted as a function of incubation time at 37°C.

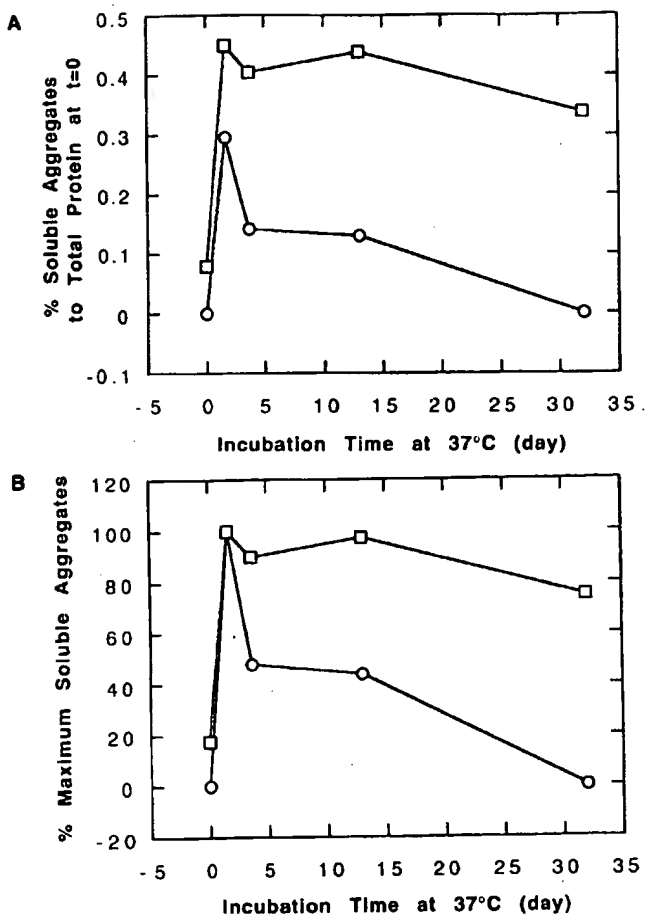

Figure 3: Kinetics of increase and decrease of soluble aggregates upon incubation at 37°C for sample C7N (○) and P7NE (□), respectively. Panel A shows soluble aggregates as a percentage of total protein at t = 0 and panel B shows soluble aggregates as a percentage of its maximum value in terms of peak area as a function of the incubation time, respectively.

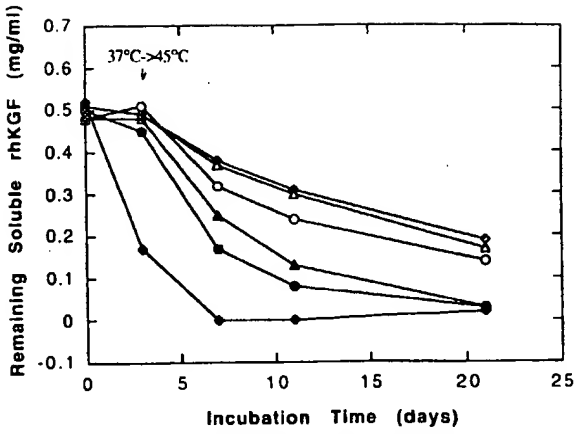

Figure 4: Kinetic plots of remaining soluble rhKGF in the presence of polymers. The concentration of rhKGF was measured by UV/Vis absorbance. Samples were first incubated at 37°C for 3 days and then transferred to 45°C for the rest of the experiment. Samples are: (♦) for the control, (○) for 0.5 mg/ml poly(acrylic acid), (●) for 0.05 mg/ml poly(acrylic acid), (△) for 0.5 mg/ml poly(methacrylic acid), (▲) for 0.05 mg/ml poly(methacrylic acid), and (◇) for 5 mg/ml heparin.

Preventing rhKGF Aggregation

Effect of Polyanions

KGF is known as a heparin-binding protein. Protein conformational stabilization through specific ligand binding is a well known strategy (16-18). Heparin is a sulfated polysaccharide and rhKGF is a highly positively charged protein (3). To test if stabilization of rhKGF by heparin is a result of charge-charge interaction, other negatively charged polymers such as poly(acrylic acid) and poly(methacrylic acid) were also studied. These two anionic polymers are organically synthesized and therefore are not "natural" KGF ligands. Figure 4 shows the effect of heparin and the two anionic polymers on the amount of soluble rhKGF remaining in solution as a function of incubation time at elevated temperatures (first at 37°C for 3 days and subsequently at 45°C for the rest of the experiment). In this experiment, heparin with an average molecular weight of 16,500 daltons, poly(acrylic acid) with an average molecular weight of 10,000 daltons and poly(methacrylic acid) with an average molecular weight of 15,000 daltons were chosen. The control contained 0.5 mg/ml rhKGF, 20 mM sodium phosphate and 150 mM NaCl at pH 7. The heparin sample contained 5 mg/ml heparin. The four anionic polymer samples contained either 0.5 mg/ml or 0.05 mg/ml of these two polymers, respectively.

The polyanions all showed pronounced stabilizing effect on rhKGF by preventing aggregation. At 37°C, the control sample had already lost about 70% of the soluble rhKGF over three days, while the samples containing the polyanions showed almost no loss. At 45°C, the control sample rapidly lost all the remaining soluble rhKGF (The real loss of soluble rhKGF was much more rapid than shown in the figure because the first time point was taken at 4 days after shifting from 37°C to 45°C). Samples containing polyanions showed much slower loss rates of soluble rhKGF. Thus aggregation of rhKGF is greatly suppressed by these polyanions. 5 mg/ml heparin and 0.5 mg/ml poly(methacrylic acid) have a similar stabilizing effect on rhKGF protein and are more effective than 0.5 mg/ml poly(acrylic acid). The stabilizing effect of these anionic polymers depends on their concentration. Higher concentrations are more effective in stabilizing rhKGF (Figure 4).

In addition to heparin, 5 mg/ml sulfated polysaccharides such as dextran sulfate (MW 8,000 daltons and 500,000 daltons) and pentosan polysulfate are also found to effectively prevent rhKGF protein aggregation. After incubation at 37°C for 1 month, little or no loss of soluble rhKGF is observed, while the control (containing no sulfated polysaccharides) and samples containing 5 mg/ml unsulfated dextrans (MW 39,100 daltons and 515,000 daltons) show a 2-3 day half-life for loss of soluble rhKGF (19).

Stabilization of rhKGF by Small Buffer Ions

Stabilization of rhKGF by small buffer ions was investigated in imidazole (I7N), sodium phosphate (P7N) and sodium citrate (C7N). Three samples were prepared to contain

Table I. Half-life for Remaining Soluble rhKGF in Samples I7N (Imidazole), P7N (Sodium Phosphate) and C7N (Sodium Citrate) Stored at 37°C. All Samples Contain 0.5 mg/ml rhKGF, 10 mM Buffer and 140 mM NaCl at pH 7

Sample	Half-life at 37°C
I7N	1.1 days
P7N	1.8 days
C7N	4.3 days

0.5 mg/ml rhKGF, 10 mM buffer and 140 mM NaCl at pH 7. Loss of soluble rhKGF protein in these samples upon incubation at 37°C was monitored by IEX-HPLC. The half-lives are estimated from the kinetics of the remaining soluble rhKGF and are listed in Table 1. Roughly, citrate is two-fold more effective than phosphate and phosphate is two-fold more effective than imidazole in preventing rhKGF aggregation.

Effect of Citrate Concentration

The stabilizing effect of citrate on rhKGF was further investigated by varying its concentration. Formulation samples were prepared to contain 0.5 mg/ml rhKGF, 100 mM to 500 mM sodium citrate and 50 mM NaCl at pH 7. The amount of soluble rhKGF protein remaining after incubation at 45°C was analyzed by both IEX-HPLC and SEC-HPLC. The half-lives of the remaining soluble rhKGF were estimated from kinetic curves of soluble rhKGF as a function of incubation time. The half-lives are then plotted as a function of the citrate concentration (Figure 5). The half-life of the remaining soluble rhKGF protein increases with increasing citrate concentration. The stabilizing effect of citrate strongly depends on its concentration.

Sulfated Polysaccharides Partially Protect rhKGF from Acid Inactivation

The rhKGF protein is rapidly inactivated at acidic conditions. Figure 6 shows rhKGF *in vitro* bioactivity measured by the mitogenic bioassay (see material and methods) in the presence and absence of 5 mg/ml dextran sulfate (MW 500,000 daltons) as a function of pH from pH 1.6 to 7. In both cases, the bioactivities are normalized to their values at neutral pH, respectively.

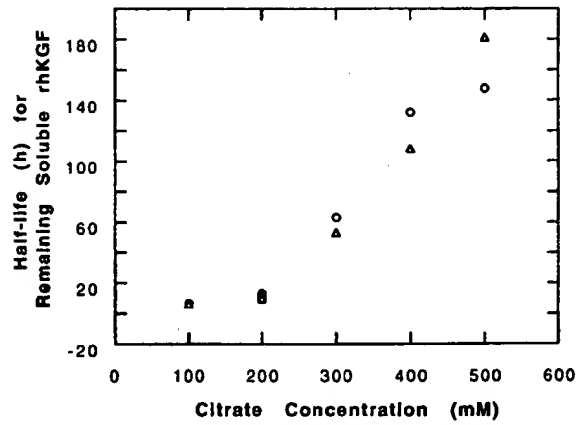

Figure 5: Dependence of half-life of remaining soluble rhKGF on citrate concentration at 45°C. Remaining soluble rhKGF was quantified by both IEX-HPLC (○) and SEC-HPLC (△), respectively.

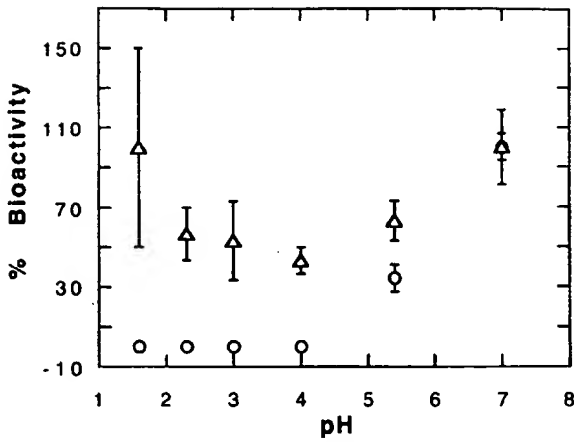

Figure 6: Effect of pH on *in vitro* bioactivity of rhKGF. *In vitro* bioactivities of rhKGF in the presence (△) and absence (○) of 5 mg/ml dextran sulfate MW 500,000 daltons are presented as percentage of their pH 7 values, respectively.

The *in vitro* bioactivity of rhKGF decreases as the pH decreases. At pH 5.5, about 35% bioactivity is observed with respect to its value at pH 7. Below pH 4, rhKGF loses all of its bioactivity. In the presence of dextran sulfate, the shape of the pH-bioactivity curve remains unchanged, i.e., rhKGF bioactivity decreases with pH. However, about half of the bioactivity is still retained below pH 4. Thus, rhKGF is partially protected from acid inactivation by dextran sulfate.

Heparin is also found to be effective in stabilizing rhKGF under acidic conditions. In the presence of heparin, no loss of rhKGF was observed by SDS-PAGE after 2 h incubation at pH 2.3 at 37°C. On the other hand, while both poly(acrylic acid) and poly(methacrylic acid) show a great stabilizing effect on rhKGF under neutral pH conditions, they have a poor stabilizing effect on rhKGF under acidic conditions (not shown).

DISCUSSION

Preventing protein aggregation is one of the major concerns in formulation of proteins because aggregation inactivates proteins and may cause immunoresponse (20-21). Aggregation is usually found to be either noncovalent *via* hydrophobic interaction or covalent *via* thiol-disulfide interchanges. Because different proteins may aggregate by different pathways, preventing aggregation of a protein requires a specific strategy designed for that molecule.

A hypothesis for the rhKGF aggregation mechanism can be proposed based on our results. Native rhKGF first unfolds, possibly because of physical instability due to charge repulsion (see below). The unfolded polypeptide chains associate to form non-covalent soluble aggregates. These aggregates are then converted to precipitates which can be readily dissolved by reducing SDS sample buffer. And finally these dissolvable precipitates (possibly simple disulfide-linked precipitates) are turned to precipitates which can not be readily dissolved by reducing SDS sample buffer (possi-

bly scrambled disulfide-linked and/or non-disulfide cross-linked precipitates). This proposed aggregation pathway is shown in Equation 1.

$$\text{Native} \rightleftharpoons \text{Unfolded} \xrightarrow[\text{reversible?}]{} \text{Soluble aggregates (little or no disulfide cross-links)} \xrightarrow[\text{not reversible}]{} \text{Precipitates SDS-reducing agent dissolvable} \longrightarrow \text{Precipitates SDS-reducing agent non-dissolvable} \quad (1)$$

Elucidation of the aggregation pathway will shed light on the rational design of formulation screens. Aggregation induced by physical instability can be prevented by addition of protein conformational stabilizers. Aggregation caused by thiol-disulfide scrambling can be alleviated by antioxidants or metal ion chelaters.

In our studies, heparin, sulfated polysaccharides, anionic polymers, and citrate can greatly reduce the rate of rhKGF protein aggregation by 50-100 fold (see Fig. 5 and reference 19). On the other hand, EDTA, a metal chelating agent, can only stabilize rhKGF moderately. Stability studies of several rhKGF analogs, in which cysteine residues are replaced by serine residues, indicate that the rate of aggregation is decreased several fold (B-L. Chen, E. Hsu and W. C. Kenney, unpublished results).

The stabilizing effects of heparin, sulfated polysaccharides, and anionic polymers on the rhKGF protein seem to be predominantly by means of electrostatic interaction. At neutral pH, these stabilizers all contain negative charges, while the rhKGF protein is strongly positively-charged. Interaction of these stabilizers with rhKGF greatly reduces the rate of aggregation at elevated temperatures. However, two unsulfated dextrans that contain no negative charges show no stabilizing effect on rhKGF.

We propose that the instability of rhKGF is probably caused by repulsion between positively charged residues brought into close vicinity by the native conformation. These closely packed positively charged residues may form the heparin binding sites. Interaction with polyanions can decrease the repulsion and thus stabilize the native protein conformation. We have found that heparin and dextran sulfate can stabilize rhKGF under acidic conditions whereas poly(acrylic acid) and poly(methacrylic acid) can not. This is probably a result of protonation of negatively-charged carboxylic groups on these two polymers under acidic conditions (22).

Stabilization of rhKGF by small buffer ions appears to depend on the number of negative charges on these ions. Imidazole has a pKa, 7.0; phosphoric acid has three pKas, 2.1, 7.2 and 12.3; and citric acid has three pKas, 3.1, 4.7 and 6.4. At neutral pH conditions, the imidazole should carry zero or one positive charge, most of the phosphate ions should carry one or two negative charges and most of the citrate ions should carry three negative charges. To interact with positively charged residue clusters on the protein surface, an ion containing more negative charges is expected to be more effective. Our results show that the rank of these buffers in stablizing rhKGF is consistent with the number of negative charges on the buffer ions (Table 1). Therefore, charge-charge interaction between the protein and the stabilizers may be the main determinant factor in stabilization.

Poly(methacrylic acid) is more effective than poly(acrylic acid) in stabilizing rhKGF. It is possible to speculate that the bulky methyl groups on the polymer backbone make poly(methacrylic acid) more extended than poly(acrylic acid), and thus have better contacts with the rhKGF protein in a charge-charge interaction.

The evidence presented previously shows that rhKGF aggregates formed immediately following unfolding seems to contain little or no inter-molecular disulfides. Intermolecular disulfides and disulfide scrambling are developed at later stages of the aggregation pathway. Thus interactions in the initial soluble protein aggregates seem to be predominantly physical in nature. This may explain why heparin, sulfated polysaccharides, poly(acrylic acid), poly(methacrylic acid), and citrate all have significant stabilizing effects on rhKGF, while EDTA has a moderate effect on the stability of rhKGF. Heparin, sulfated polysaccharides, poly(acrylic acid), poly(methacrylic acid), and citrate are all polyanions or negatively charged molecules. Their stabilizing effects on rhKGF appear to be through electrostatic interaction, which stabilizes the native conformation and prevents the exposure of the hydrophobic patches involved in the formation of soluble rhKGF protein aggregates. These molecules may provide their beneficial effects on the stability of rhKGF because they act at the first step (native $\rightleftharpoons$ unfolded form) in the aggregation pathway as proposed in Eq. 1. On the other hand, EDTA, which inhibits the later steps (soluble aggregates $->$ precipitates), can only provide a moderate stabilizing effect on rhKGF. A mechanistic approach to the study of protein aggregation has helped us to begin to better understand the process of aggregate formation and to search for better stabilizers.

ACKNOWLEDGMENTS

We thank Dr. Susan Hershenson for comments on the manuscript during its preparation and Lynn Wilson for performing the KGF *in vitro* bioassay.

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